

# Palmitoylation of TNF alpha is involved in the regulation of TNF receptor 1 signalling

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## ABSTRACT

The pleiotropic pro-inflammatory cytokine tumour necrosis factor alpha (TNF) is synthesised as a transmembrane protein that is subject to palmitoylation. In this study, the roles of this acylation on TNF-mediated biological effects were investigated. We found that the lipid raft partitioning of TNF is regulated by its palmitoylation. Furthermore, we demonstrated that this palmitoylation process interferes with the cleavage/degradation of TNF intracellular fragments but is not involved in the regulation of its ectodomain shedding. Moreover, we found that the palmitoylation of TNF hinders the binding of soluble TNF to TNFR1 and regulates the integration/retention of TNFR1 into lipid rafts. Finally, we demonstrate that the transmembrane forms of wild-type and palmitoylation-defective TNF interact differently with TNFR1 and regulate NFκB activity, Erk1/2 phosphorylation and interleukin-6 synthesis differently, strongly suggesting that palmitoylation of TNF is involved in the regulation of TNFR1 signalling. An evidence for the physiological intervention of this regulation is provided by the fact that, in macrophages, the binding of endogenous soluble TNF to TNFR1 is enhanced by inhibition of palmitoylation. Therefore, our data introduce the new concept that palmitoylation of TNF is one of the means by which TNF-producing cells regulate their sensitivity to soluble TNF.

## Keywords:

TNF  
TNFR1  
Lipid rafts  
Palmitoylation  
Signalling

## 1. Introduction

Tumour necrosis factor alpha (TNF) is a pro-inflammatory cytokine that exhibits a wide range of biological effects. TNF is synthesised as a type II transmembrane protein (tmTNF). Upon stimulation, the TNF alpha-converting enzyme ADAM17 cleaves the extracellular domain of tmTNF, which releases the soluble TNF (sTNF) [1]. The newly generated membrane-bound moiety of TNF is further cleaved within its intramembrane region by signal peptide peptidase-like 2b (SPPL2b), leading to the generation of an intracellular domain (ICD) of TNF [2].

Soluble TNF signals through two distinct cell surface receptors, TNFR1 and TNFR2. Most of the biological activities of TNF are mediated through TNFR1 [3]. Upon binding to TNFR1, the ligand-bound receptor aggregates and serves as a scaffold to recruit adaptor proteins [4]. The activated submembranous complex triggers cellular activation via NFκB and mitogen-activated protein kinases or apoptosis via complex internalisation and activation of apical caspases.

In recent years, it has become clear that sTNF is not solely responsible for all of the effects of TNF. Indeed, tmTNF elicits biological activities, some of which are similar to those of sTNF and some of which are distinct [5–8]. These biological effects involve the binding of TNF to its specific receptors and downstream signal transduction either by the transmembrane receptors (forward signalling) or by tmTNF (reverse signalling) [9,10]. Furthermore, the ICD might possess biological effects; its overexpression has been shown to stimulate the activity of the interleukin-1β (IL1β) promoter and to trigger interleukin-12 (IL12) expression [11,12]. Therefore, it is plausible that TNF-producing cells are subject to the simultaneous actions of tmTNF, sTNF and ICD.

The diversity of TNF-mediated signalling responses is facilitated by lipid rafts. The two TNF receptors are partially embedded in lipid rafts [13–15], and the destruction of these membrane microdomains alters the cellular response to TNF [16,17]. It has been proposed that depending on whether TNFR1 signalling emanates from inside or outside lipid rafts, it can induce strong or weak NFκB activation, respectively (reviewed in [18,19]). Furthermore, the dynamic distribution of specific transmembrane proteins between lipid raft and non-raft phases has been demonstrated to influence the regulation of their functions [20]. This applies to TNF signalling; upon sTNF binding, the TNF–TNFR1 complex translocates into lipid rafts, where it is internalised and initiates proapoptotic signals

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[21,22]. Therefore, the response to TNF depends on how the cell integrates signals emanating simultaneously from different membrane domains.

Although its biological significance remains unknown, a significant amount of tmTNF localises within lipid rafts [15,23,24]. Palmitoylation of the juxtamembrane cytoplasmic Cys30 of tmTNF [25] was suggested to be responsible for the targeting of TNF into lipid rafts, as a non-palmitoylable mutant form of TNF is not integrated into these membrane microdomains [24].

Our study aims to evaluate the impact of TNF palmitoylation on its regulation and function. We found that this palmitoylation is necessary for both the lipid raft partitioning of TNF and the processing of its cellular fragments but is dispensable for the shedding of its ectodomain. Importantly, we demonstrated that palmitoylated TNF interferes with the binding of sTNF to TNFR1 and regulates TNFR1 integration/retention in lipid rafts, phenomena that involve a physical interaction between TNFR1 and palmitoylated TNF. As a result, TNF palmitoylation contributes to the regulation of TNFR1 signalling.

## 2. Materials and methods

### 2.1. Reagents

The general metalloproteinase inhibitor GM6001, 2-bromohexadecanoic acid (bromopalmitate), phorbol 12-myristate 13-acetate (PMA), methyl- $\beta$ -cyclodextrin and anti-Flag M2 affinity agarose were from Sigma-Aldrich. The cleavable bifunctional crosslinker dimethyl 3,3'-dithiobispropionimidate (DTBP) was from Pierce. The signal peptide peptidase inhibitor (Z-LL)2-ketone and the MEK inhibitor PD098059 were from Calbiochem. Human recombinant soluble TNF and mouse recombinant soluble TNF were from Peprotech. Antibodies against TNF (N-19), TNFR1 (H-5), actin (C-11), caveolin-1 (N-20), HA epitope (Y-11), Erk2 (D-2), p-Erk (E-4) and NF $\kappa$ B p65 (A) were from Santa Cruz Biotechnology. Antibodies against ADAM17 (AB930; polyclonal; discontinued), TNF (clone 1825 for blocking and clone 6401 for flow cytometry) and TNFR1 (clone 16803 for blocking and flow cytometry) were from R&D Systems. The DYKDDDDK epitope tag (Flag) polyclonal antibody (PA1-984B) was from Pierce. Antibodies against I $\kappa$ B $\alpha$  (9242) and p-NF $\kappa$ B p65 (93H1) were from Cell Signaling Technologies.

### 2.2. Expression vectors

Human TNF and TNFR1 expression vectors were described previously [26]. The non-palmitoylable form of TNF ( $\Delta$ palTNF) was generated by substituting Cys30 with Ala (N-terminal amino acid = position 1). A Flag tag (DYKDDDDK) was added at the N-terminal position of the different forms of TNF. Expression vectors for active and inactive forms of HA-tagged SPPL2b were described previously [27]. The mouse ADAM17-expression vector was provided by J. Peschon [11].

### 2.3. Cell culture and transfection

Hela, 3T3L1, ADAM17-deficient (ADAM17<sup>zn/zn</sup>) and RAW264.7 cell lines were cultured as previously described [28–30]. Transient transfections were performed with Polyjet reagent (SignaGen Laboratories), as specified by the manufacturer's protocols. For all cotransfections, an empty plasmid was used to keep the amount of DNA constant. For stable expression of TNF, Hela cells were transfected with linear vectors using Polyjet reagent. Forty-eight hours after transfection, 1 mg/mL of G418 sulphate was added, and the cells were subjected to a limiting dilution procedure. Two independent clones expressing comparable levels of TNF and  $\Delta$ palTNF at their surface and releasing comparable levels of sTNF (Table 1) were chosen. These clones were then cultured in the presence of 300  $\mu$ g/mL of G418 sulphate. ICD was detected

using the previously established and validated model of HEK 293 cells overexpressing SPPL2b [31].

### 2.4. Cell surface binding and internalisation of sTNF

Hela cells were washed with ice-cold PBS, washed twice for 2 min in an acidic buffer (50 mM glycine pH 3, 100 mM NaCl), and then washed again with PBS. Recombinant murine sTNF (100 ng/mL) was added to the cells and incubated for 1 h on ice, and then the cells were extensively washed with PBS. To measure the cell surface binding of sTNF, the cells were lysed at this stage using 1% Triton X 100 in PBS. For the measurement of sTNF internalisation, the cells were incubated at 37 °C for 1 h, washed twice with the acidic buffer and then lysed. The murine sTNF contained in cell lysates was measured by ELISA and the results are shown as the means  $\pm$  s.d. normalised to the control situation.

### 2.5. Isolation of lipid rafts

Low density detergent-resistant membrane domains, referred to herein as lipid rafts, were isolated by sucrose density gradient centrifugation of cells treated with the non-ionic detergent Brij 98, as described previously [15]. Briefly, 5 to 10  $\times$  10<sup>6</sup> cells were washed with ice-cold PBS, harvested by scraping, collected by centrifugation, and resuspended in 900  $\mu$ L of "raft buffer" (20 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EGTA, with protease, phosphatase and metalloproteinase inhibitors). Lysis was performed by equilibrating the cell suspension at 37 °C, followed by the addition of Brij 98 to a final concentration of 1%. The incubation was carried out at 37 °C for 10 min. One millilitre of the lysate was combined with an equal volume of 90% (w/v) sucrose, transferred to 12 mL ultracentrifuge tubes, overlaid with a discontinuous sucrose gradient composed of 4 mL of 35% sucrose, and further overlaid with 2 mL of 5% sucrose. Separation of the low-density lipid rafts was achieved by ultracentrifugation at 280,000 g in a swinging bucket rotor for 18–22 h at 4 °C. Following ultracentrifugation, 0.5 mL fractions were harvested from the top of the gradient.

### 2.6. Detection of TNF palmitoylation

Detection of TNF palmitoylation was performed by click chemistry [32], in which, incorporated azido-palmitate was reacted with biotin-alkyne (Life Technologies). Briefly, Hela cells were washed twice with serum free media and incubated for 12 h in the labelling media containing 0.7% fatty-acid free BSA and 70  $\mu$ M of azido-palmitate. Cells were washed and either lysed directly or submitted to lipid raft isolation. Pools of lipid raft and non-raft fractions containing comparable amounts of TNF were collected. The protein content and composition of the pools were equilibrated by adding a pool of non-raft fractions from control cells to the pool of raft fractions from TNF expressing cells; reciprocally, a pool of raft fractions from control cells was added to the pool of non-raft fractions from TNF expressing cells. After chloroform/methanol precipitation, proteins were reacted by click chemistry with biotin-alkyne,

**Table 1**  
release and cell surface expression of TNF.

	sTNF released			Cell surface tmTNF
	Hela	3T3L1	Hela clones	Hela clones
Control	6.38 $\pm$ 0.6	nd	0.12 $\pm$ 0.03	1 $\pm$ 0.11
TNF	380 $\pm$ 21	3.48 $\pm$ 0.57	0.90 $\pm$ 0.05	6.41 $\pm$ 0.72
$\Delta$ palTNF	378 $\pm$ 30	3.28 $\pm$ 0.70	0.98 $\pm$ 0.09	6.34 $\pm$ 0.61

Cells that transiently (Hela, 3T3L1) or stably (Hela clones) express TNF or  $\Delta$ palTNF and their control were incubated for 14 h in fresh culture media. sTNF accumulated in the media was measured by ELISA and expressed as pg/ $\mu$ g of total cellular protein ( $\pm$  s.d.). Cell surface expression of tmTNF was measured by flow cytometry and normalised to the control situation as described in the Materials and methods section. nd = not detected.

as described by the manufacturer (Life Technologies). Biotinylated proteins were enriched by neutravidin-agarose (Thermo Pierce) binding and analysed by western blot using TNF antibody (N-19).

## 2.7. Immunoprecipitation

For immunoprecipitation of native ectopically expressed proteins, cells were washed with PBS and then lysed in lysis buffer (50 mM Hepes, pH 7.4, 5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 1% Triton X 100 (v/v), protease and phosphatase inhibitors). Lysates were preclarified by centrifugation and incubated for 4 h with Flag M2 affinity agarose. After incubation, the agarose was pelleted by centrifugation, washed 3 times, boiled in SDS-PAGE sample buffer and submitted to immunoblot analysis. For immunoprecipitation of crosslinked ectopically expressed proteins, the cells were washed with PBS, incubated for 30 min at room temperature with the crosslinker DTBP (5 mM), washed twice with 50 mM Tris HCl, pH 7.4, and then lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 0.1% SDS, 150 mM NaCl). Lysates were treated for immunoprecipitation as described above.

## 2.8. Purification of cell surface protein

The cell surface protein isolation kit from Pierce that uses the cell-impermeable, cleavable biotinylation reagent, sulfo-NHS-SS-biotin, was used according to the manufacturer's protocols. Equal amounts of total proteins from each sample were purified on NeutrAvidin agarose resin.

## 2.9. Immunoblot

Equal amounts of protein from each cell lysate were resolved under denaturing and reducing conditions on 4–12% NuPAGE gels (Life Technologies) and then transferred to polyvinylidene fluoride membranes (Millipore). Immunoreactive proteins were revealed by enhanced chemiluminescence with ECL (Perkin-Elmer). Images were captured using a CCD camera (ImageQuant™ LAS 4000 system; GE Healthcare), and the intensity of the bands was evaluated by the image analysis software ImageQuant™ TL.

## 2.10. ELISA assays

The human TNF and interleukin-6 (IL6) ELISA kits were from Life Technologies and eBioscience, respectively. The human and mouse TNFR1 TNF ELISA kits were from R&D Systems. The TNFR1–TNF heterodimer immunoassay was made by combining the capture antibody from the mouse TNFR1 ELISA kit and the detection antibody from the mouse TNF ELISA kit. The phospho-Erk1/2 ELISA kit was from RayBiotech.

## 2.11. Immunofluorescence microscopy

Cells cultured on microscopy chamber slides (Labtek) were treated for immunofluorescence detection of Flag-tagged TNF. Briefly, cells were washed, fixed on ice with 4% paraformaldehyde for 10 min, permeabilised with 0.2% Triton X 100 in PBS and saturated for non-specific binding with 3% BSA for 30 min. After 1 h of incubation with the anti-Flag antibody, cells were incubated with a secondary antibody coupled to Alexa Fluor 546. Cells were mounted in a DAPI-containing medium and visualised using a fluorescence microscope (Leica DMRB). Images were captured using a camera (ProgRes CFcool; Jenoptik, Germany) and ProgRes Capture Pro software (version 2.6). Images were prepared for presentation using ImageJ software with minimal processing.

## 2.12. Flow cytometry analysis

Cells were washed with PBS and subject or not to two washes of 2 min with the acidic buffer, followed by a subsequent PBS wash, and detached by Accutase treatment. Surface expression of tmTNF and TNFR1 was determined by flow cytometric analyses using PE-conjugated (clone 6401) and APC-conjugated (clone 16803) antibodies, respectively. Labelled cells were analysed on the Accuri C6 flow cytometer. The means ± s.d. of fluorescence intensities were recorded and for ease of reading, values were corrected for the value obtained with an isotype control and normalised to the control situations.

## 2.13. Gene reporter experiments/luciferase assay

Cells were cotransfected with an NFκB-firefly luciferase reporter (Panomics; Fremont, CA, USA) and a renilla luciferase control (PGL4 sv40 from Promega) vector. Firefly and renilla luciferase activities were measured using a luminometer (Victor X; Perkin Elmer). The transcriptional activity of NFκB is shown as the ratio of the arbitrary units of luminescence of firefly versus renilla luciferase and normalised to controls.

## 2.14. Real time PCR analysis

Total RNA was extracted using the PureLink RNA Mini Kit (Life Technologies). RT-PCR was performed using a 7300 real-time PCR system (Applied Biosystems) using the Eva Green MasterMix (Euromedex). The relative levels of the different mRNAs were measured with the comparative CT method ( $2^{-(\Delta\Delta CT)}$ ). Acidic ribosomal phosphoprotein P0 (36B4) was used as an internal housekeeping control transcript. Primer sequences are provided in Supplementary Table S1.

## 2.15. Caspase 8 assay

Caspase 8 activity was measured with a colorimetric assay kit according to the manufacturer's protocols (PromoKine). The assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labelled substrate IETD-pNA. The pNA light emission was quantified using a microtiter plate reader at 405 nm.

## 2.16. Statistical analyses

All experiments were performed in triplicates and repeated, at least, three times. All data are expressed as the mean ± s.d. Treatments were compared with their respective controls, and significant differences among the groups were determined using the unpaired Student's *t*-test. A value of  $p < 0.05$  was considered statistically significant. Results obtained with the independent Hela clones were very close and for ease of reading, they were combined in the statistical analysis.

# 3. Results

## 3.1. Palmitoylation of TNF promotes its lipid raft integration

A long period of accumulation was necessary to measure trace amounts of sTNF in culture media of mock transfected Hela cells (Table 1), whereas tmTNF was undetectable in their lysates (Fig. 1D). Overexpression of TNF greatly increased sTNF levels and was mandatory for the detection of tmTNF (Table 1 and Fig. 1D).

The small amounts of tmTNF detected in lipid rafts of TNF overexpressing Hela cells were significantly reduced by a treatment with the general palmitoylation inhibitor bromopalmitate (Fig. 1A), which did not notably affect the distribution of the lipid raft marker caveolin-1. This result implies that the incorporation of TNF into



lipid rafts requires a palmitoylation process. Furthermore, palmitoylated tmTNF was preferentially detected inside lipid rafts (Fig. 1B). This result highlights the importance of TNF palmitoylation in the regulation of its lipid raft partitioning. Moreover, a palmitoylation-defective form of TNF ( $\Delta$ palTNF) (Fig. 1B) was less integrated into lipid rafts than wild-type TNF (Fig. 1C, D), proving that the localisation of TNF in lipid rafts requires its specific palmitoylation. Despite this difference in lipid raft integration, the cell surface expression (Fig. 1E and Table 1) and the general cellular localisation of overexpressed TNF and  $\Delta$ palTNF (Fig. 1F) were similar.

### 3.2. Palmitoylation of TNF is not essential for the cleavage of its extracellular domain

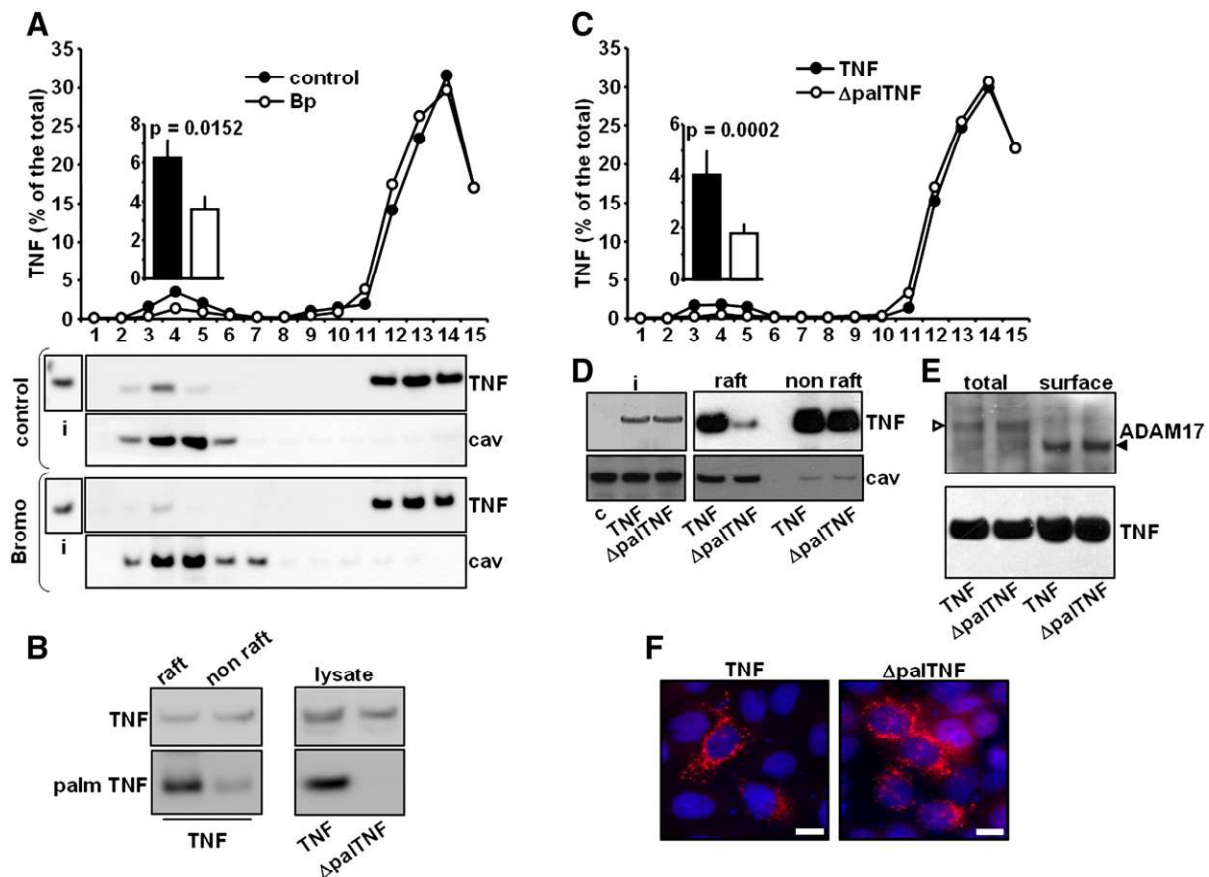
We analysed whether TNF palmitoylation participated in the regulation of its ectodomain shedding.

ADAM17-deficient ( $\text{ADAM17}^{\text{Zn/Zn}}$ ) mouse fibroblasts transiently overexpressing TNF or  $\Delta$ palTNF released comparable levels of sTNF, which were increased to similar extents by the simultaneous overexpression of ADAM17 and by PMA treatment (Fig. 2A). Similarly, overexpression of TNF or  $\Delta$ palTNF in Hela or 3T3L1 cells led to the release of comparable amounts of sTNF (Fig. 2B and Table 1). These results indicate that the palmitoylation of TNF is minimally or not involved in the regulation of ADAM-17-dependent cleavage of its ectodomain.

However, the shedding of TNF and  $\Delta$ palTNF was comparably stimulated by disruption of lipid rafts (Fig. 2B) suggesting that the integrity of lipid rafts is required for the proper regulation of TNF shedding.

### 3.3. Palmitoylation of TNF is required for the correct processing of its intracellular fragments

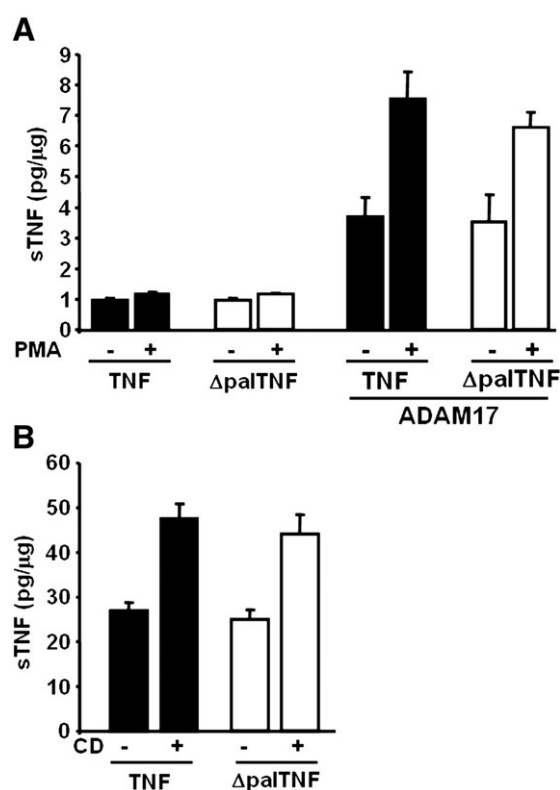
Notably, even if the levels of full-length TNF and  $\Delta$ palTNF overexpressed in  $\text{ADAM17}^{\text{Zn/Zn}}$ , Hela and 3T3L1 cells were comparable, the N-terminal fragment of TNF (NTF), which remains membrane-anchored after the cleavage of the ectodomain, was more present in TNF-overexpressing cells (Fig. 3A,B). In  $\text{ADAM17}^{\text{Zn/Zn}}$  cells, stimulation of TNF shedding by overexpression of ADAM17 (Fig. 2A) reduced the levels of full-length tmTNF and increased the levels of its NTF (Fig. 3A). As expected, activation of ADAM17-dependent shedding of TNF by a short exposure to PMA (Fig. 2A), reduced the levels of full-length TNF and increased the levels of its NTF (Fig. 3A, B). In contrast, the NTF of the  $\Delta$ palTNF was almost undetectable under similar conditions. Furthermore, we showed that the amount of NTF derived from full-length TNF was reduced by bromopalmitate, whereas the amount of NTF derived from  $\Delta$ palTNF was not altered by this treatment (Fig. 3C). However, bromopalmitate treatment similarly reduced the shedding of TNF and  $\Delta$ palTNF (Fig. 3D). Altogether, these data imply that palmitoylation of TNF stabilises its NTF.



**Fig. 1.** Palmitoylation of TNF contributes to its lipid raft integration. (A) Hela cells stably expressing TNF were treated or not for 14 h with 100  $\mu$ M bromopalmitate (Bp) then submitted to lipid raft isolation as indicated in the Materials and methods section. TNF contained in the fractions of the sucrose density gradient (numbered according to increasing sucrose density) was measured by ELISA and expressed as the percentage of the total levels present in the gradient. TNF and the lipid raft marker caveolin-1 (cav) were also detected by western blot. (B) Palmitoylated TNF (palm TNF) was measured as described in the Materials and methods section in lipid raft and non-raft fractions of Hela cells stably expressing TNF and in lysates of Hela cells expressing TNF or  $\Delta$ palTNF. (C) Hela cells transfected with TNF or  $\Delta$ palTNF were treated for lipid raft isolation and TNF was measured by ELISA in the fractions of the sucrose density gradient. Then lipid raft fractions (3–7) and non-raft fractions (8–15) were pooled, concentrated and TNF and caveolin-1 (cav) were detected by immunoblot (D). Insets in panels A and C are positioned above lipid raft fractions and show the % of TNF contained in those fractions (p values are indicated). (E) Immunoblot detection of total and cell surface exposed TNF,  $\Delta$ palTNF and ADAM17. Mature and pro-forms of ADAM17 are indicated by the black and white arrowheads, respectively. (F) Flag-tagged TNF and  $\Delta$ palTNF overexpressed in Hela cells were detected by immunocytochemistry using an anti-Flag antibody and epifluorescence microscopy (red); nuclei were stained with DAPI (blue); bars = 10  $\mu$ m. i: cell lysate before fractionation; c: cells transfected with empty plasmid.



Membrane-anchored NTF is normally cleaved by the intramembrane protease SPPL2b, whereupon the ICD is released. As expected, the overexpression of active SPPL2b reduced the levels of NTF detected (Fig. 4A and Fig. S1). However, treatment of cells overexpressing  $\Delta$ palTNF with a pharmacological inhibitor of signal peptide peptidase did not restore the levels of NTF comparable to those observed in cells overexpressing TNF (Fig. 4B). Moreover, the increased levels of NTF induced by long-term inhibition of signal peptide peptidases were consistently greater in cells overexpressing TNF ( $+40\pm 6\%$ ) than in those overexpressing  $\Delta$ palTNF ( $+13\pm 7\%$ ). Furthermore, ICD was not detectable in  $\Delta$ palTNF-overexpressing cells (Fig. 4C and Fig. S1). These results indicate that the NTF derived from  $\Delta$ palTNF ectodomain shedding is rapidly degraded and suggest that its degradation is not only attributed to proteolysis by SPPL2b. We further evaluated the stability of the NTF generated from the two forms of TNF. For this purpose, we blocked NTF generation by inhibiting TNF ectodomain shedding (Fig. 4D) using a metalloproteinase inhibitor active on ADAM17 (GM6001), and we analysed NTF levels after 5 h of inhibitor treatment. The NTF generated from TNF was relatively stable over this short period of time (Fig. 4E) and the inhibition of signal peptide peptidases did not elicit any apparent effects. Conversely, the inhibition of  $\Delta$ palTNF ectodomain shedding was accompanied by a reduction in the levels of its NTF ( $-30\pm 7\%$ ), indicating its active degradation. This degradation was not blocked by the inhibition of signal peptide peptidases (surprisingly, it was even slightly increased). Taken together, these results highlight that the palmitoylation of TNF is essential for the stabilisation of its NTF and for its subsequent, efficient cleavage by SPPL2b.



**Fig. 2.** Palmitoylation of TNF is not essential for its extracellular shedding. Soluble TNF (sTNF) was measured by ELISA in the culture media of (A) ADAM17<sup>Zn/Zn</sup> cells transfected with TNF or  $\Delta$ palTNF in association or not with ADAM17 then stimulated or not for 30 min with 100 nM of PMA and (B) Hela cells transfected with TNF or  $\Delta$ palTNF incubated for 30 min with 1% of methyl- $\beta$ -cyclodextrin (CD). Values are shown in pg/μg of protein contained in the cell lysates.

### 3.4. The cleavage of NTF by SPPL2b occurs preferentially in lipid rafts

As previously described [33], we found that a large proportion of overexpressed SPPL2b localised inside lipid rafts (Fig. S2A) and we analysed whether the SPPL2b-mediated cleavage of NTF occurs in lipid raft. Inhibition of signal peptide peptidase activity efficiently increased the levels of NTF present within the lipid rafts and modestly increased the levels of NTF outside the lipid rafts (Fig. S2B). These results indicate that SPPL2b more efficiently cleaves NTF that is partitioned inside the lipid rafts.

### 3.5. Palmitoylation of TNF modulates TNF responses

Since palmitoylation of TNF regulates several aspects of its biology (lipid raft partitioning, degradation and generation of its intracellular fragments), we analysed whether this acylation could also change its biological activity.

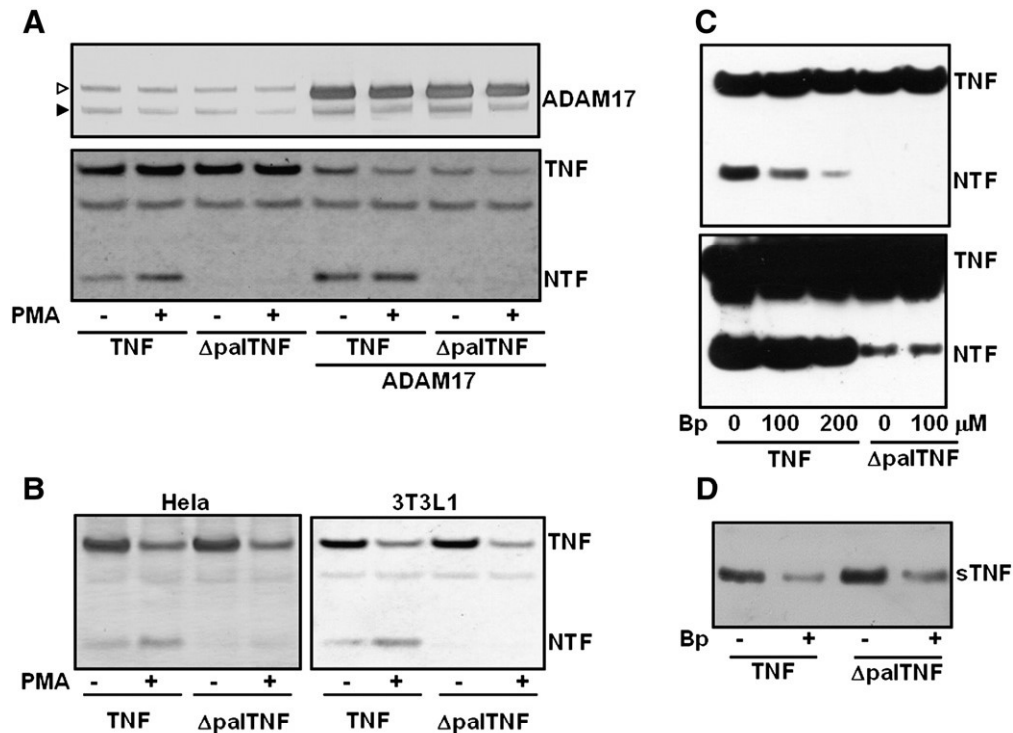
#### 3.5.1. The NF $\kappa$ B target genes

We investigated whether wild type and palmitoylation-deficient forms of TNF elicited differential effects on cellular responses triggered by bioactive TNF. For this purpose, we first analysed the expression of several TNF target genes (plasminogen activator inhibitor-1, *I $\kappa$ B*, *IL6*, *TNF* and *IL1 $\beta$* ) in Hela cells that transiently overexpressed TNF or  $\Delta$ palTNF. This model was used to highlight the strong effects of tmTNF (as compared to sTNF). Indeed, only a subset of cells (30 to 40%) overexpressed tmTNF and produced significant levels of sTNF (Table 1), which acts on all the cells. Among TNF target genes whose expression was measured, only *IL6* was repeatedly and significantly expressed to higher levels in Hela cells that transiently overexpressed  $\Delta$ palTNF compared to TNF-overexpressing cells (Fig. S3A). However, although significant, this difference of expression was rather low, therefore this result was verified and reinforced in stably transfected Hela cells that are overexpressing comparable levels of transmembrane TNF and  $\Delta$ palTNF at their surface and releasing comparable levels of sTNF (Table 1);  $\Delta$ palTNF expressing cells synthesised more *IL6* than control and TNF-expressing cells (Fig. 5A, B). Consistently, *IL6* mRNA levels were increased in mouse fibroblasts by 64% and 31% by the transient overexpression of  $\Delta$ palTNF and TNF, respectively (Fig. S3B). Stimulation with sTNF more efficiently increased *IL6* synthesis in control cells compared to Hela cells that expressed TNF or  $\Delta$ palTNF (Fig. 5A, B).

The overexpression of the ICD of TNF in Hela cells has been reported to increase *IL1 $\beta$*  promoter activity [11]. As TNF and  $\Delta$ palTNF result in the production of significantly different levels of ICD (Fig. 4C), we analysed how these two forms of TNF might regulate *IL1 $\beta$*  expression. Overexpression of TNF and  $\Delta$ palTNF similarly increased the expression of *IL1 $\beta$* , which was reduced by the addition of a TNF-blocking antibody but not by the inhibition of signal peptide peptidase activity (Fig. S3A). These results indicate that the palmitoylation of TNF is likely not involved in the regulation of *IL1 $\beta$*  expression and that *IL1 $\beta$*  expression is principally regulated by the bioactive TNF and minimally by the ICD.

#### 3.5.2. The NF $\kappa$ B signalling pathway

To better understand the disparity in *IL6* expression levels, we analysed the impact of TNF and  $\Delta$ palTNF on NF $\kappa$ B signalling. The stable expression of TNF and  $\Delta$ palTNF in Hela cells decreased the levels of *I $\kappa$ B* and increased the phosphorylation and the nuclear levels of NF $\kappa$ B (Fig. S4A). A short stimulation with sTNF strongly increased NF $\kappa$ B phosphorylation and decreased *I $\kappa$ B* levels in control cells but did not significantly elicit such effects in TNF- or  $\Delta$ palTNF-expressing cells. These results indicate that TNF expression interferes with the cellular response to sTNF. Quantitative analysis revealed that  $\Delta$ palTNF expression was more effective than TNF expression in increasing NF $\kappa$ B activity ( $+86\%$  and  $+48\%$ , respectively, compared to control cells) (Fig. 5C). Furthermore, the increased NF $\kappa$ B activity triggered by



**Fig. 3.** Palmitoylation of TNF is required for the correct processing of its cellular fragments. The N-terminus of TNF was detected by immunoblot analysis (using the anti-Flag antibody) in lysates of (A) ADAM17<sup>Zn/Zn</sup> cells transfected with Flag-tagged TNF or ΔpalTNF in association or not with ADAM17 then stimulated with PMA, (B) HeLa and 3T3L1 cells transfected with Flag-tagged TNF or ΔpalTNF then stimulated with PMA, (C) HeLa cells transfected as in (B) and incubated for 14 h with the indicated concentrations of bromopalmitate (Bp) (upper panel: short exposure; lower panel: long exposure). (D) Western blot detection of sTNF accumulated in the media of HeLa cells overexpressing TNF and ΔpalTNF treated or not with bromopalmitate (BP; 100 μM for 14 h). TNF indicates full-length tmTNF; NTF indicates tmTNF fragment (generated by the ectodomain cleavage of TNF). Mature and pro-forms of ADAM17 are indicated by the black and white arrowheads, respectively.

treatment with sTNF was low but significant in control cells, marginal in TNF-expressing cells and non-existent in ΔpalTNF-expressing cells. Similarly, the transient overexpression of human TNF and ΔpalTNF in mouse fibroblasts reduced IκB levels and increased NFκB phosphorylation (Fig. S4B). Luciferase reporter experiments revealed that TNF and ΔpalTNF overexpression increased NFκB activity by 23% and 55%, respectively, compared to control cells (Fig. S4C). As for HeLa cells, the increased NFκB activity triggered by sTNF treatment was low but significant in control cells (+27%), marginal in TNF-overexpressing cells and non-existent in ΔpalTNF-overexpressing cells.

### 3.5.3. The Erk1/2 signalling pathway

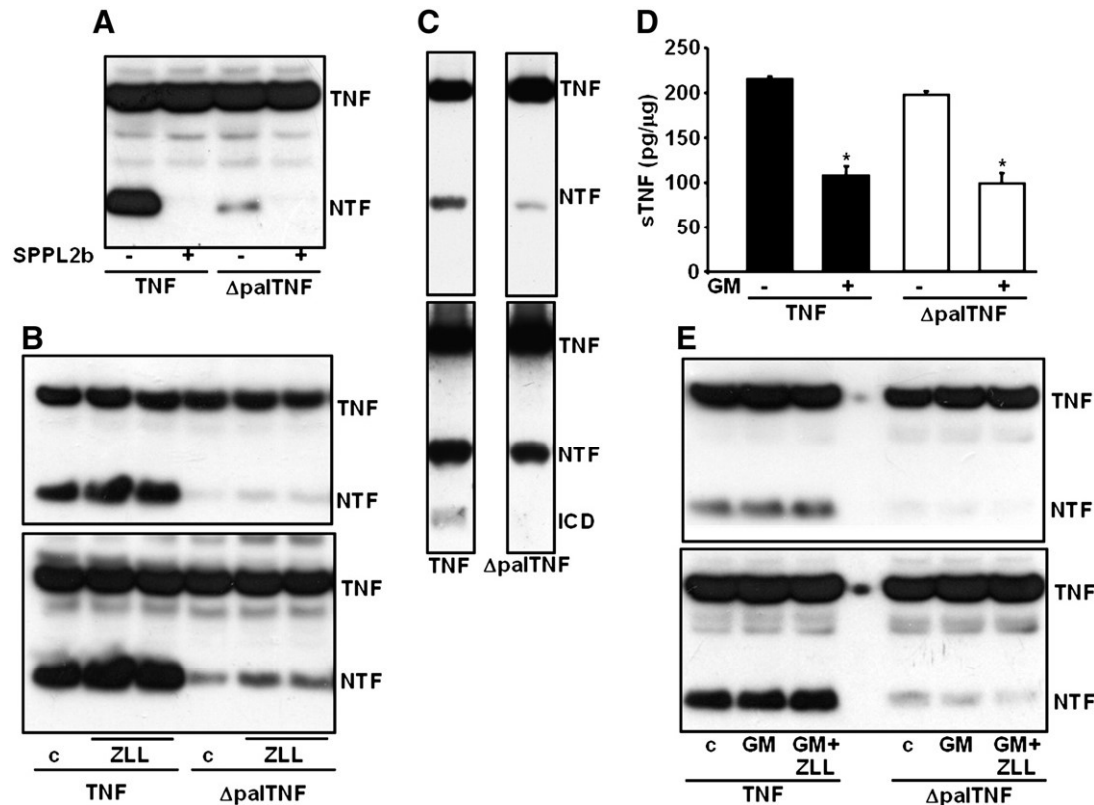
TNF activates the Erk1/2 signalling pathway [16,34], and the inhibition of Erk1/2 was sufficient to reduce the stimulatory effect of sTNF on IL6 expression (Fig. S3C). Thus, we analysed the effects of TNF and ΔpalTNF expression on Erk1/2 phosphorylation. Stimulation of control HeLa cells with sTNF triggered a rapid increase in Erk1/2 phosphorylation, which persisted for at least 20 min (Fig. S5D). This pattern of phosphorylation was not significantly different from that observed in TNF transiently overexpressing HeLa cells. The basal level of Erk1/2 phosphorylation in ΔpalTNF-expressing HeLa cells was more than twice that measured in control and TNF-expressing cells, and furthermore, it was not stimulated by sTNF treatment. Western blot analysis suggested that Erk2 was primarily responsible for the measurable increase in Erk1/2 phosphorylation (Fig. S5E). An elevated level of Erk2 phosphorylation was also observed in murine fibroblasts that transiently overexpressed ΔpalTNF (Fig. S4D).

Taken together, these data demonstrate that TNF and ΔpalTNF differently regulate NFκB and Erk1/2 signalling, which could explain their differential ability to stimulate IL6 synthesis.

### 3.6. Palmitoylation of TNF modulates the binding of sTNF to TNFR1

TNFR1 represents the vast majority of TNFRs in HeLa cells [35] and we verified that TNFR2 expression was not altered by TNF overexpression (Fig. S5A). Importantly, TNFR1 is responsible for the TNF-dependent activation of NFκB, Erk1/2 pathways and IL6 synthesis [16,34] and human TNF exclusively signals through TNFR1 in mouse cells [36]. These observations support the possible involvement of TNFR1 in the differential regulation of NFκB, Erk1/2 and IL6 induced by TNF and ΔpalTNF.

Thus, we evaluated the binding of sTNF to TNFR1 in TNF- and ΔpalTNF-expressing cells. The ability of our assay to measure the binding of sTNF to TNFR1 was validated by the observation that the cell surface binding of sTNF was increased by the overexpression of TNFR1 and reduced by the addition of a TNFR1-blocking antibody (Fig. 6A). TNF-expressing cells bound less sTNF than control and ΔpalTNF-expressing cells (Fig. 6A, B). These results indicate that TNF palmitoylation is involved in the control of sTNF binding to the cell surface. However, after an acid wash, to remove any receptor-bound ligand, TNF- and ΔpalTNF-expressing cells bound similar levels of sTNF (Fig. 6B), suggesting that the binding of sTNF to TNFR1 is specifically impaired in TNF-expressing cells. Unexpectedly, this binding was similar to that obtained with control cells that expressed more TNFR1 at their surface (Fig. S5B). Although the specific role of palmitoylation of endogenous TNF cannot be analysed in cells that express TNF physiologically, we nevertheless determined whether binding of sTNF to TNFR1 was effectively regulated by a palmitoylation process. RAW264.7 mouse macrophages treated with PMA (to increase TNF synthesis) and with an inhibitor of ADAM17 (to reduce TNF release) were incubated or not with bromopalmitate. Bromopalmitate treatment did not change the amount of cellular TNFR1 and TNF (Fig. 6C)



**Fig. 4.** NTF derived from  $\Delta$ palTNF undergoes SPPL2b-independent degradation. (A) HeLa cells were transfected with Flag-tagged TNF or  $\Delta$ palTNF associated with inactive (–) or active (+) forms of SPPL2b. (B) HeLa cells were transfected with Flag-tagged TNF or  $\Delta$ palTNF and treated for 14 h with 20  $\mu$ M (Z-LL)2-ketone (ZLL). (C) HEK 293 cells stably expressing active SPPL2b were transfected as in (B). The entire immunoblot analysis is shown in Fig. S1. (D) HeLa cells transiently transfected with TNF or  $\Delta$ palTNF were treated or not with 10  $\mu$ M GM6001 (GM) for 5 h. The accumulation of sTNF in the culture media was measured by ELISA. Values are shown in pg/ $\mu$ g of protein in the cell lysates. (E) HeLa cells were transfected as in (B) and treated for 5 h with 10  $\mu$ M GM6001 (GM) or with GM + ZLL. TNF was detected as described in Fig. 3. Upper panels: short exposure; lower panels: long exposure. ICD indicates intracellular domain of TNF generated by the action of SPPL2b. \*Significant versus the situation without GM6001 ( $p < 0.006$ ).

but reduced that of their soluble forms (Fig. 6D). However, more endogenous sTNF was bound at the cell surface after bromopalmitate treatment (Fig. 6E). Using a home-made TNFR1–TNF heterodimer immunoassay we demonstrated that bromopalmitate treatment increased the amount of TNF bound to cellular TNFR1 (Fig. 6F). These results argue in favour of the role of a palmitoylation process in the regulation of sTNF binding to the TNFR1 of cells expressing TNF physiologically.

### 3.7. Palmitoylation of TNF alters its interaction with TNFR1

Lipid rafts from control and TNF-expressing cells contained more TNFR1 than those from  $\Delta$ palTNF-expressing cells (Fig. 7A, B), indicating that the palmitoylation of TNF is involved in the regulation of the partitioning of TNFR1 in lipid rafts. The difference in the levels of TNFR1 in lipid rafts cannot be explained by its altered internalisation (Fig. S6A) or shedding (Fig. S6B), as both events were similarly reduced in TNF- and  $\Delta$ palTNF-overexpressing cells compared to control cells. Moreover, TNFR1 was more efficiently coimmunoprecipitated with full-length TNF than with  $\Delta$ palTNF (Fig. 7C, D). This result was observed with both non-crosslinked proteins obtained from the lysis of the cells using a mild, non-ionic detergent (Fig. 7C), as well as crosslinked proteins obtained from an aggressive cell lysis (Fig. 7D). These results suggest that TNFR1 interacts more efficiently with TNF than with  $\Delta$ palTNF and that this interaction occurs in lipid rafts.

### 3.8. Palmitoylation of TNF does not modify Caspase 8 activation

TNFR1-dependent apoptosis is initiated by the activation of the apical Caspase 8 [37,38]. However, we did not observe any difference in Caspase 8 activity in cells expressing TNF or  $\Delta$ palTNF (Fig. S7),

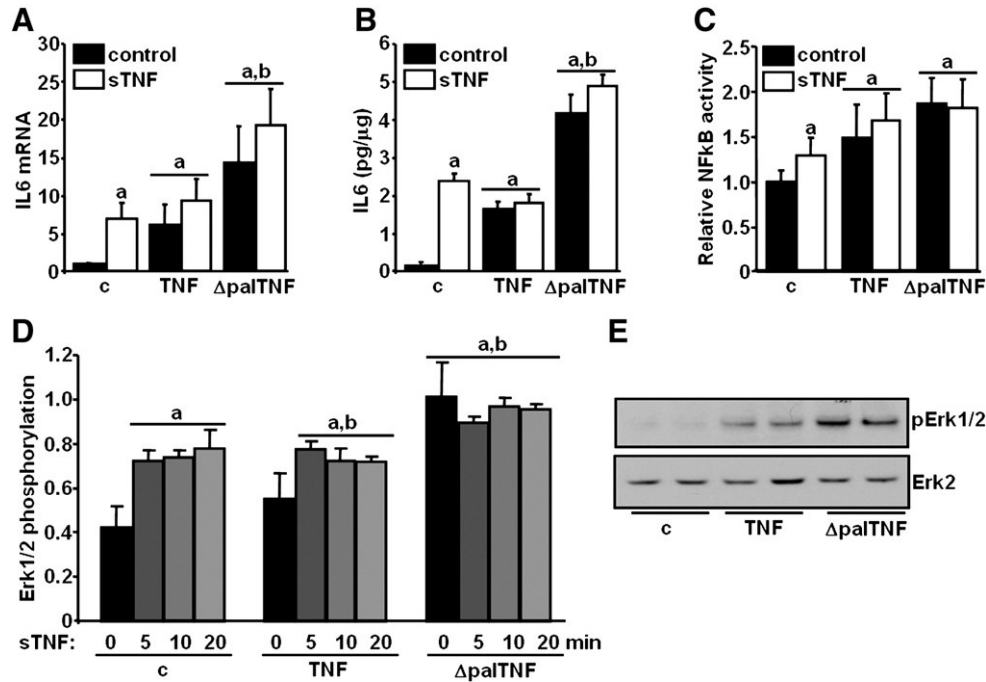
suggesting that certain aspects of TNFR1 signalling are not altered by TNF palmitoylation.

## 4. Discussion

Palmitoylation of TNF was first described more than ten years ago [25]. However, the functional role of this particular acylation in TNF biology and function was unknown. As a reversible lipid modification, cysteine palmitoylation is considered a dynamic lipid raft-targeting mechanism for several transmembrane proteins [39]. Our results demonstrate that the palmitoylation of TNF largely contributes to its recruitment to lipid rafts without interfering with its intracellular localisation or its cell surface expression. However, a small proportion of palmitoylated TNF was also detected outside lipid rafts. We previously reported that the mature form of ADAM17 is present both inside and outside of lipid rafts and that its inhibition resulted in increased TNF levels within lipid rafts [15]. This led to the hypothesis that the extracellular domain of TNF is preferentially cleaved by ADAM17 in these membrane microdomains. However, our current results suggest that the role of lipid rafts in the regulation of the TNF ectodomain shedding is more complex than originally anticipated, as both wild-type and palmitoylation-deficient TNF ( $\Delta$ palTNF) are similarly cleaved by ADAM17. Furthermore, we and others have proposed that lipid raft disruption increased ADAM17 substrate shedding by allowing their cleavage outside lipid rafts [15,40]. As wild-type TNF and  $\Delta$ palTNF are mostly contained outside lipid rafts, it is therefore not surprising that disruption of lipid rafts increases their release.

The palmitoylation of TNF is involved in the control of the intracellular degradation of the transmembrane moiety generated by its ectodomain shedding (i.e., NTF). Indeed, the NTF derived from TNF is relatively stable





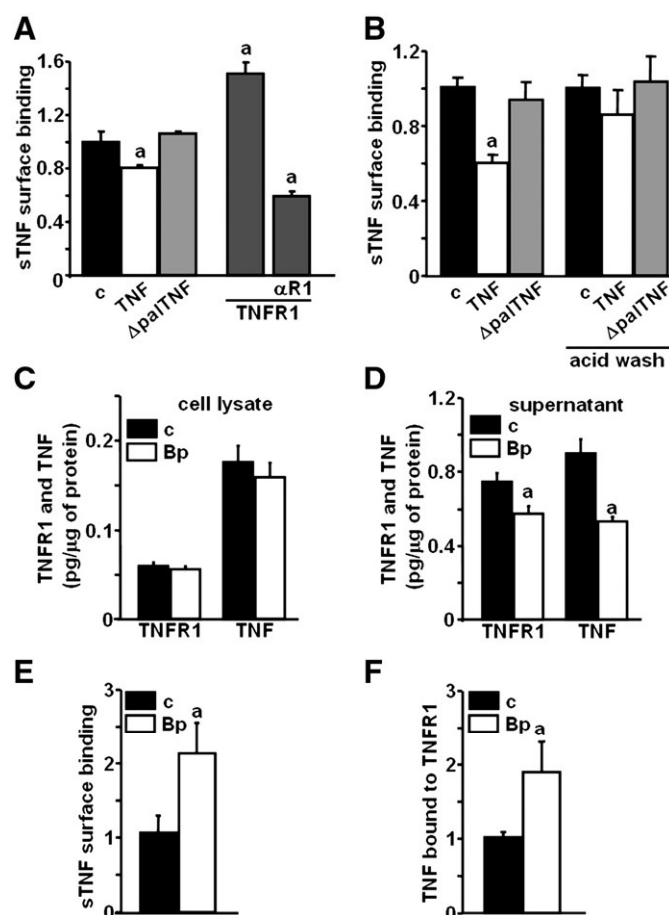
**Fig. 5.** TNFR1 signalling is differentially altered by TNF and  $\Delta$ palTNF. Stably transformed Hela cells were treated or not for 14 h with sTNF (50 ng/mL), IL6 mRNA levels were measured by RT-PCR (A) and the accumulation of IL6 protein in the culture media was measured by ELISA (B). Values are shown as pg/ $\mu$ g of protein in the cell lysate. (C) Stably transformed Hela cells were transfected with NF $\kappa$ B luciferase reporter, stimulated or not with sTNF, and NF $\kappa$ B activation was measured as described in the [Materials and methods](#) section. The results are shown as the ratio of firefly luciferase activity/renilla luciferase activity and normalised to the controls. (D) Phosphorylated and total Erk1/2 were measured by ELISA in the total lysates of Hela cells that stably expressed the empty vector (c), TNF or  $\Delta$ palTNF and that were stimulated for the indicated periods of time with sTNF (50 ng/mL). The results show pErk1/2 signal (OD 450 nm) normalised to the total Erk1/2 signal. (E) immunoblot of total Erk2 and phosphorylated Erk1/2. a – significant versus cells transfected with empty vector (c) without sTNF treatment ( $p < 0.03$ ); b – significant versus cells overexpressing TNF without sTNF treatment ( $p < 0.02$ ).

and is a substrate for SPPL2b, which generates the intracellular N-terminal fragment of TNF (ICD). The NTF generated by the ectodomain shedding of  $\Delta$ palTNF is unstable (independent of the action of SPPL2b), consequently impairing the detection of its derived ICD. However, this NTF is nonetheless a substrate for SPPL2b because its levels are reduced upon SPPL2b overexpression and are stabilised by treatment with a pharmacological inhibitor of signal peptide peptidases. Thus, the increased degradation of the ICD derived from  $\Delta$ palTNF cannot be excluded as a possible explanation for the lack of its detection. Notably, the increased levels of NTF derived from TNF processing induced by the pharmacological inhibition of signal peptide peptidases are modest compared to the dramatic loss of NTF resulting from SPPL2b overexpression. These data suggest that in our cell model, NTF is not efficiently cleaved by SPPL2b. Consistent with previous studies, we report that SPPL2b is contained predominantly in lipid rafts [33]. The colocalisation of TNF and SPPL2b in lipid rafts might explain why the NTF derived from TNF is a better substrate for SPPL2b than the NTF derived from  $\Delta$ palTNF, which is largely excluded from lipid rafts. The use of different inhibitors of intracellular proteolytic systems did not enable us to identify the system responsible for the accelerated degradation of NTF in cells that overexpressed  $\Delta$ palTNF. Similar technical difficulties were reported previously in a study of accelerated degradation of the non-palmitoylated form of the C-terminal fragment of betacellulin (a  $\gamma$ -secretase substrate) [41]. The NTF generated by TNF ectodomain shedding is detected in ADAM17-deficient cells, indicating that an ADAM17-independent cleavage of TNF occurs in these cells, which is also illustrated by their ability to release sTNF. However, the re-expression of ADAM17 reduces the levels of full-length TNF, increasing the levels of NTF and proving that ADAM17 activity participates in the production of the NTF.

ICD overexpression was shown to stimulate the activity of the IL1 $\beta$  promoter in Hela cells and to trigger IL12 expression in activated human dendritic cells [11,12]. However, despite the fact that TNF and  $\Delta$ palTNF lead to the production of significantly different amounts of ICD they similarly increase IL1 $\beta$  mRNA levels (the expression of

IL12 analysed by RT-PCR was below the detection limit), an effect that is predominantly attributed to the bioactivity of TNF. The transcriptional changes induced by the ICD generated from NTF cleavage warrant further investigation, as they might provide evidence for potential specific effects of TNF and  $\Delta$ palTNF.

Expression of TNFR1 at the surface of endothelial cells was shown to be down-regulated by treatment with sTNF [42]. Furthermore, overexpression of a non-secretable tmTNF mutant in L929 cells resulted in a total TNFR1 down-modulation and induction of TNF unresponsiveness [43], while in Hela cells, overexpression of a non-secretable tmTNF did not significantly affect TNFR1 surface expression [44]. In our Hela cell models, the chronic expression of TNF and  $\Delta$ palTNF merely halves the total and cell surface levels of TNFR1. This reduction of TNFR1 expression is likely not attributed to its shedding or internalisation (as both were reduced). Interestingly, the difference in TNFR1 expression at the surface of control and TNF-expressing cells is not reflected by a difference in the binding of sTNF to TNFR1, suggesting that sTNF binding to TNFR1 is not only regulated by the levels of TNFR1 expressed at the cell surface. However, although TNF and  $\Delta$ palTNF-expressing cells expose similar amounts of tmTNF at their surface and release comparable levels of sTNF, our results indicate that less sTNF binds to TNFR1 at the surface of TNF-expressing cells compared to  $\Delta$ palTNF-expressing cells (and control cells). An impediment of sTNF binding at the surface of Hela cells that overexpress a non-secretable tmTNF was previously reported and explained by the formation of a complex between tmTNF and TNFR1 [44]. Our results are consistent with this explanation; indeed we showed that TNFR1 is more efficiently coimmunoprecipitated with tmTNF than with tm $\Delta$ palTNF. As the lipid rafts from TNF-overexpressing cells contain more TNFR1 than those from  $\Delta$ palTNF-overexpressing cells, the coimmunoprecipitation of TNF and TNFR1 might be explained either by an interaction between the two proteins or by the precipitation of TNF with all or part of the lipid raft domains containing TNFR1. However, after crosslinking and lipid raft lysis, TNFR1 is still more efficiently



**Fig. 6.** Palmitoylation of TNF modulates the binding of sTNF to TNFR1. sTNF binding assays were performed as described in the [Materials and methods](#) section on (A) HeLa cells transiently transfected with TNF, ΔpalTNF or TNFR1 and incubated for 1 h with 10 μg/mL of TNFR1-blocking antibody (αR1); (B) control and stably TNF- or ΔpalTNF-expressing HeLa cells (untreated or acid-washed). Endogenous TNF and TNFR1 were measured by ELISA in the lysates (C) and culture media (D) of RAW264.7 cells treated for 18 h with PMA (20 nM) and TMI-1 (5 μM) in absence or presence of bromopalmitate (Bp; 100 μM). Endogenous sTNF bound at the surface of RAW264.7 cells was measured after its release by the acid wash of the cells (E). Endogenous sTNF bound to RAW264.7 TNFR1 was measured in cell lysate using a home-made heterodimer immunoassay described in the [Materials and methods](#) section (F). a — significant versus control (c) ( $p < 0.02$ ).

coimmunoprecipitated with tmTNF than with tmΔpalTNF, suggesting a closer proximity between TNF and TNFR1 than between ΔpalTNF and TNFR1. This result is consistent with a favoured interaction between TNF and TNFR1, which does not preclude the possibility that this interaction occurs in lipid rafts. An indication of the physiological involvement of TNF palmitoylation in the regulation of its interaction with TNFR1 is provided by the observation that global inhibition of palmitoylation by treating macrophages (that physiologically express TNF) with bromopalmitate increases the binding of sTNF to TNFR1. Incidentally, it should be noticed that bromopalmitate reduces the shedding of TNF as well as that of the non-palmitoylated proteins ΔpalTNF and TNFR1 [45]; this highlights the limitations of using bromopalmitate to unambiguously conclude on the role of the palmitoylation of a specific protein and emphasizes the need for approaches involving the expression of palmitoylation deficient form of the studied protein.

TNFR1 has been detected in lipid rafts during basal conditions [13,14,42], and the engagement of TNFR1 with sTNF has been shown to lead to a transient increase (within minutes) [14] or to a net decrease [42] in the levels of TNFR1 in lipid rafts. Our models of TNF-producing cells are chronically exposed to the released sTNF, and we assessed the steady state levels of TNFR1 in lipid

rafts. Control and TNF-expressing cells contain comparable levels of TNFR1 in lipid rafts, which weakens the hypothesis that TNFR1 is driven into lipid rafts by a potential interaction with tmTNF. As TNFR1 and tmΔpalTNF do not exhibit a strong interaction, the low levels of TNFR1 in lipid rafts of tmΔpalTNF-expressing cells cannot be explained by its retention outside lipid rafts due to an interaction with tmΔpalTNF. It is conceivable that tmΔpalTNF, which is sparsely represented in lipid rafts, interacts poorly with TNFR1 and does not impair the binding of sTNF to the receptor. As a consequence, the sTNF/TNFR1 complex is normally downregulated in lipid rafts. Conversely, palmitoylated tmTNF interacts with TNFR1 in lipid rafts, which blocks the binding of sTNF to a subset of receptors; the palmitoylated tmTNF/TNFR1 complex is not downregulated. The low level of TNFR1 blocked by tmTNF in lipid rafts compared to the total levels of TNFR1 might explain why we did not observe decreased internalisation of sTNF in TNF-overexpressing cells compared to those expressing ΔpalTNF. In agreement with such a difference in the biology of TNFR1 in TNF- and ΔpalTNF-expressing cells, we showed that some aspects of post-TNFR1 signalling are differentially affected. Indeed, basal NFκB activity and Erk1/2 phosphorylation are higher in ΔpalTNF-expressing cells than in control and TNF-expressing cells. Furthermore, stimulation with sTNF increased NFκB activity and Erk1/2 phosphorylation only in control and TNF-expressing cells but not in cells expressing ΔpalTNF. Elevated NFκB activity and Erk1/2 phosphorylation in ΔpalTNF expressing cells might explain the lack of responsiveness of these cells to sTNF. These alterations in the signalling downstream of TNFR1 affect the cellular response to TNF, which is illustrated by differential expression levels of IL6 (an NFκB-target gene) in TNF- and ΔpalTNF-expressing cells. Importantly, constitutive activation of NFκB and IL6 production associated with a reduced response to sTNF was already described in HeLa cells overexpressing non-secretable tmTNF [44]. Why, in our study, the expression of the other NFκB-target genes studied did not exhibit expression changes similar to that observed in IL6 remains unclear. However, it is conceivable that the relative proportion of the effects of NFκB and Erk1/2, and perhaps the effects of other factors not yet identified, are involved in this regulation. Furthermore, the expression of TNF and ΔpalTNF did not alter all aspects of TNFR1 signalling. Indeed, we did not notice differences in Caspase 8 activation.

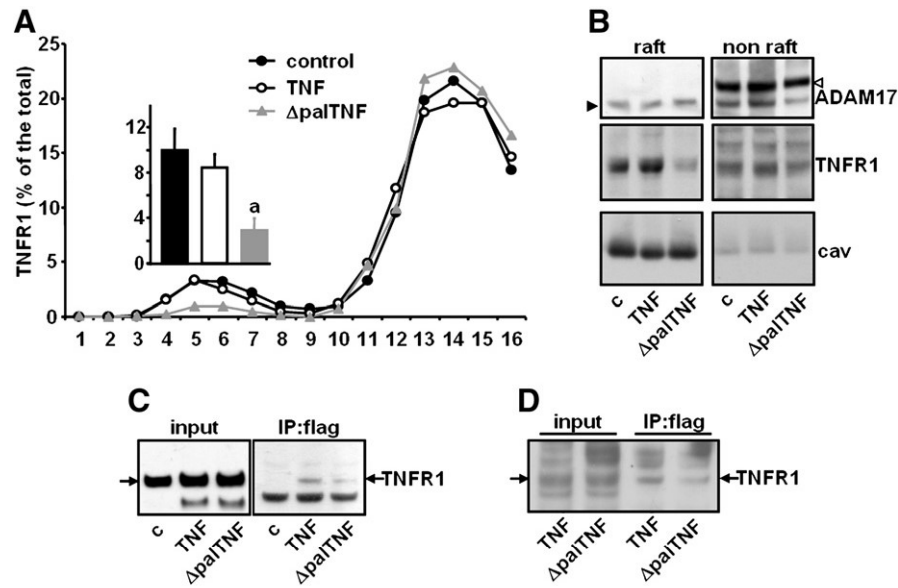
To integrate our results in the physiological context of TNF-producing cells, one must consider that at any time, tmTNF exists in two forms: one that is non-palmitoylated, and another that is palmitoylated. Furthermore, these cells are permanently subjected to the effects of the sTNF they produce. The non-palmitoylated form of TNF (outside lipid rafts) would have no substantial effect on the biology of TNFR1, whereas the palmitoylated form (inside lipid rafts) diminishes TNFR1 signalling by interacting with the receptor and by reducing sTNF binding. The fact that inhibition of palmitoylation in macrophages enhanced the binding of endogenous sTNF to TNFR1 provides an evidence for the physiological implication of this regulation. Therefore, our data introduce the new concept that palmitoylation of TNF is a means by which TNF-producing cells regulate their sensitivity to sTNF.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamcr.2012.11.009>.

## Acknowledgements

The authors are indebted to F. Brun and L. Rebsomen (Aix-Marseille Université) for their valuable contribution in lipid raft purification and the cloning of tagged forms of TNF.

This work was funded by INSERM, INRA and Aix-Marseille Université. MP is a recipient of the European Society of Cardiology. IK is a recipient of the Ministère de la Recherche et de l'Enseignement Supérieur.



**Fig. 7.** Palmitoylation of TNF alters its interaction with TNFR1. (A) Lysates from control and stably TNF- and  $\Delta$ palTNF-expressing HeLa cells were fractionated by sucrose density gradient centrifugation and TNFR1 was measured by ELISA in each fraction. Inset positioned above the lipid raft fractions shows the % of TNF contained in those fractions (a — significant versus control and TNF-expressing cells ( $p = 0.0036$ )). (B) Lipid raft and non-raft fractions were pooled and concentrated, and TNFR1, caveolin-1 (cav) and ADAM17 were detected by immunoblot analysis. HeLa cells transfected with TNFR1 alone (c) or together with Flag-tagged TNF or  $\Delta$ palTNF were lysed with Triton X 100 buffer (C) or treated with DTBP to crosslink the proteins and lysed with RIPA buffer (D). Then, TNF was immunoprecipitated with anti-Flag antibody (IP:flag) and TNFR1 was detected by immunoblot analyses. Mature and pro-forms of ADAM17 are indicated by the black and white arrowheads, respectively. Arrow indicates TNFR1 position.

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